



Published in final edited form as:

Eur J Immunol. 2016 May ; 46(5): 1152–1161. doi:10.1002/eji.201546094.

Follicular regulatory T cells repress cytokine production by follicular helper T cells and optimize IgG responses in mice

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Abstract

Follicular helper T (T_{fh}) cells provide crucial help to germinal center B (GCB) cells for proper antibody production, and a specialized subset of regulatory T cells, follicular regulatory T (T_{fr}) cells, modulate this process. However T_{fr}-cell function in the GC is not well understood. Here, we define T_{fr} cells as a CD4⁺ Foxp3⁺ CXCR5^{hi} PD-1^{hi} CD25^{low} TIGIT^{high} T-cell population. Furthermore, we have used a novel mouse model (“Bcl6^{FC}”) to delete the Bcl6 gene in Foxp3⁺ T cells and thus specifically deplete T_{fr} cells. Following immunization, Bcl6^{FC} mice develop normal T_{fh}- and GCB-cell populations. However, Bcl6^{FC} mice produce altered antigen-specific antibody responses, with reduced titers of IgG and significantly increased IgA. Bcl6^{FC} mice also developed IgG antibodies with significantly decreased avidity to antigen in an HIV-1 gp120 “prime-boost” vaccine model. In an autoimmune lupus model, we observed strongly elevated anti-DNA IgA titers in Bcl6^{FC} mice. Additionally, T_{fh} cells from Bcl6^{FC} mice consistently produce higher levels of Interferon- γ , IL-10 and IL-21. Loss of T_{fr} cells therefore leads to highly abnormal T_{fh}-cell and GCB-cell responses. Overall, our study has uncovered unique regulatory roles for T_{fr} cells in the GC response.

Keywords

Germinal Center Response; Follicular T cells; Regulatory T cells; Antibody; Bcl6

Introduction

During an immune response, CD4⁺ T cells can differentiate into several unique effector lineages that promote different immune responses via the secretion of distinct types of cytokines. Follicular T helper (T_{fh}) cells are a recently characterized CD4 lineage whose major function is to help B cells form germinal centers (GCs) and produce high-affinity

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Conflict of Interest Disclosure

The authors declare no financial or commercial conflict of interest.

antibodies (Abs) [1, 2]. Both in mouse and human, Tfh cells control the initiation as well as the outcome of the GCB-cell response [3-6]. While Tfh cells are critical for the proper production of Abs, the over-production of Tfh cells can lead to autoimmunity, since Tfh cells can help B cells to produce self-reactive Abs [6-8]. Thus, the proper regulation of Tfh- and GCB-cell responses is essential both for normal immune function and for preventing autoimmune disease.

A subpopulation of Foxp3+ regulatory T cells (Tregs) in the GCs was discovered that appears to act as suppressors of Tfh and GCB cells [9-11]. These regulatory follicular T cells or “Tfr” cells display a mixed phenotype of both Tfh cells and Tregs. Like Tfh cells, Tfr cells express CXCR5, ICOS, and PD-1, and depend on Bcl6 for differentiation and localization to the B cell follicle. Although Tfr and Tfh cells are phenotypically similar, Tfr cells originate from natural Tregs, whereas Tfh cells originate from naïve CD4+ T cells [10]. *In vitro* and *in vivo* studies have shown that Tfr cells can suppress Tfh- and GCB-cell proliferation and can regulate GCB-cell differentiation as well [9-13]. A study using a NFAT2 conditional knockout mouse model showed that decreased development of Tfr cells correlated with increased Tfh- and GCB-cell responses, and these mice also developed anti-dsDNA auto-Abs and lupus-like disease [14].

However how Tfr cells affect antibody (Ab) production is still unresolved. Several studies have shown that Tfr cells repress Ab production [9-13]. Furthermore, Linterman *et al* demonstrated that Tfr cells control the outgrowth of non-antigen-specific GCB cells and helped maintain high titers of high affinity antigen-specific Abs [10]. However, the precise mechanisms by which Tfr cells control Ab responses is not known.

Despite a large number of studies analyzing Tfr cells in GC responses, a mouse model where Tfr cells are specifically deleted and that allows for systematic study has been lacking. Experimental systems with adoptive transfer of Tfr cells into T cell deficient mice can have non-physiological effects [9, 10, 13, 15]. Bone marrow chimeras have been used to analyze loss of Tfr cells [10], but the number of animals that can be tested is limited and this approach relies on the production of Tfh cells from Foxp3-deficient T cells. Total Treg deletion [9-11, 14, 16, 17] does not specifically target Tfr cells, and causes a much broader effect on the immune response that can mask the actual functions of Tfr cells.

Here, we have undertaken a further characterization of Tfr cells with a novel mouse model. We find that Tfr cells have low expression of CD25 and high expression of the inhibitory molecule TIM-1. We used Bcl6^{fl/fl}Foxp3^{cre} (Bcl6FC) mice to completely delete Tfr cells and leave remaining Tregs intact. Although loss of Tfr cells has no gross effect on size of Tfh- or GCB-cell populations after immunization, we find that Tfr cells promote the generation of normal levels IgG and repress IgA responses. Further, Tfr cells repress the production of anti-dsDNA IgA Abs in a lupus model. Tfh cells from Bcl6FC mice consistently produce higher levels of IFN- γ , IL-10 and IL-21, which are well-known cytokines that promote B-cell responses. We reason that the increased cytokine production by Tfh cells in the absence of Tfr cells may lead to the altered Ab responses. Overall, our findings uncover both positive and negative regulatory roles for Tfr cells in Ab responses, both in vaccine and autoimmune settings.

Results

Characterization of Tfh and Tfr cell populations

Initially, we analyzed wild-type mice, in order to assess the development of Tfh and Tfr cells after immunization. We used Sheep Red Blood Cells (SRBC), which is known to induce a strong Tfh and GC response. As shown in Fig. 1A, both the Foxp3⁻ and Foxp3⁺ cell populations contain a fraction of cells expressing both CXCR5 and PD-1, with a gradient from intermediate expression (CXCR5^{low}PD-1^{low}) to high expression (CXCR5^{hi}PD-1^{hi}). Tfh cells are commonly defined based on the expression of these two cell surface markers [18]. For this study, in Foxp3-CD4⁺ T cells, we define CXCR5^{hi}PD-1^{hi}, CXCR5^{neg}PD-1⁺ and CXCR5^{neg}PD-1^{neg} cells as Tfh, PD-1⁺TH and PD-1⁻TH cells, respectively. We applied the same cell subpopulation gating and definition to the Treg compartment, which are Tfr, PD-1⁺Treg and PD-1⁻Tregs. Tfh and Tfr cells expressed the highest levels of Bcl6, the master transcription factor for Tfh- and Tfr-cell differentiation, among conventional CD4 T (Tconv) cells and Tregs, respectively (Fig. 1B, Supp. Fig. 1). Since IL-2 and STAT5 signaling is known to inhibit Tfh-cell differentiation [19,21] and CD25 is often used as a Treg marker, we next compared CD25 expression on different Tconv or Tregs subpopulations directly ex vivo (Fig. 1C, Supporting Figure 1). The overall level of CD25 on Tregs was much higher than Tconv cells. However, within Foxp3⁺ cells, Tfr cells had significantly lower level of CD25 than other subpopulations. This indicates that down regulation of CD25 may be part of Tfr-cell differentiation program similar to Tfh cells. Previous studies have shown that Tfh cells highly express the inhibitory molecule TIGIT [22, 23] and TIGIT is important for Treg suppressive function on Th1 and Th17 cell [24]. Therefore we checked TIGIT expression on Tfr cells. As shown in Fig. 1D (Supp. Fig. 1), both Tfh and Tfr cells express the highest level of TIGIT in Tconv and Tregs, respectively, and further that the MFI of TIGIT on Tfr cells is more than two-fold higher than Tfh cells. This is not the case for the well-known inhibitory molecule PD-1, which Tfr cells express at somewhat lower levels compared to Tfh cells (data not shown). Tfr cells are enriched within the TIGIT-high Treg population, but Tfr cells only comprise a fraction of the TIGIT-high Treg population (Supp. Fig. 2). Taken together, Tfr cells express high levels of Bcl6 and TIGIT, as well as a relatively low level of CD25. These data indicate novel differences between Tfr and Tfh cells and also between Tfr cells and conventional Tregs.

Loss of Tfr cells does not lead to an increased Tfh cell population in Bcl6FC mice

In order to assess the functional role of Tfr cells more precisely, we created Bcl6^{fl/fl}Foxp3^{cre} (Bcl6FC) mice, where floxed Bcl6 allele is deleted specifically in Foxp3 expressing T cells. Similar to Bcl6^{fl/fl}CD4^{cre} mice [25], Bcl6FC mice are healthy, fertile, do not display abnormal inflammation and have grossly normal immune cell compartments ([26] and data not shown). This is in contrast to germline Bcl6^{-/-} mice, which are extremely prone to spontaneous inflammatory disease and early death [27]. Tregs from Bcl6^{-/-} mice are strongly skewed towards a pro-inflammatory Th2 phenotype, which is largely driven by the severe Th2 inflammation in these mice [28]. However, Tregs from unimmunized Bcl6FC mice do not show abnormal Th2 cytokine production ([26] and data not shown), consistent with the lack of inflammatory disease in the mice. Prior to immunization, both control (Bcl6^{+/+}Foxp3^{cre}) mice and Bcl6FC mice have very low levels of Tfh cells, less than 0.5%

of total spleen (data not shown), indicating that the Bcl6FC mice do not have increased basal levels of Tfh cells. We then immunized control and Bcl6FC mice with SRBC and analyzed Tfh and Tfr cells in spleen 10 days post immunization (dpi). As expected, the induction of CXCR5^{hi}PD-1^{hi}Foxp3⁺ Tfr cells was strongly diminished in Bcl6FC mice (Fig. 2B, Supp. Fig. 3). We wondered the great loss of Tfr cells is due to apoptosis in the absence of Bcl6, similar to our findings in Bcl6^{fl/fl}CD4^{cre} mice [25]. In those mice, the few Tfh cells that develop are undergoing a high rate of apoptosis. We therefore examined survival of Tfr cells in Bcl6FC mice, and found that indeed, Tfr cells are undergoing a significantly higher rate of late apoptosis indicated as increased percentage of Annexin V and viability dye double positive Tfr cells (Supp. Fig. 4). However, in contrast to previous reports [9-11], we did not see an increase in the proportion or number of Tfh cells in immunized Bcl6FC mice. This result is not unique to SRBC immunization, as Bcl6FC mice also had comparable Tfh cell populations as control mice did at different time-points after immunization with SRBC, as well as in several other types of immune challenge with different antigens (data not shown). Additionally, we did not observe changes in the numbers of PD1- and CXCR5-intermediate Foxp3- T cells (early/pre-Tfh cells) in Bcl6FC mice, and thus there was no altered regulation of Tfh-cell differentiation at an earlier stage due to loss of Tfr cells (Supp. Fig. 5). Thus, Tfr cells are specifically and strongly deleted in Bcl6FC conditional KO mice, however loss of Tfr cells did not have any gross effect on the development of the Tfh-cell population.

Loss of Tfr cells does not expand the GC B-cell population but the antibody response is altered

Although we did not observe that loss of Tfr cells led to the increase in Tfh-cell numbers, we next wondered whether Tfr cells could suppress the GCB cell response. Unexpectedly, 10 days after SRBC immunization, similar sizes of GCB cell populations were formed both in control and Bcl6FC mice (Fig. 3A-B). Similar to our observations with Tfh cells, this result of unchanged GCB cell numbers in the absence of Tfr cells was observed after multiple types of immune challenge with different antigens and was seen at all time-points following immunization (data not shown). Since the ultimate outcome of GC responses is the production of antigen-specific antibodies, we next analyzed SRBC-specific IgG and IgM serum Ab titers at 10 dpi by ELISA. Despite the similar Tfh- and GCB-cell responses in control and Bcl6FC mice, we found the titers of anti-SRBC-specific IgG were strongly and significantly decreased in Bcl6FC mice, while the titers of anti-SRBC-specific IgA were strongly and significantly increased in Bcl6FC mice (Fig. 3C,D). This pattern of decreased Ag-specific IgG but increased Ag-specific IgA was also observed when the mice were immunized with NP-KLH in Alum (Fig. E,F). This indicates that Tfr cells are important for promoting high levels of IgG and repressing abnormal IgA responses.

Loss of Tfr cells decreases the avidity of antigen-specific antibodies in an HIV immunization model

Generation of high affinity, protective antibodies is one of the goals of an effective vaccine. We therefore sought to determine whether Tfr cells have an impact on the GC and antigen-specific Ab responses in a vaccination setting. We used a well-characterized DNA prime-protein boost vaccine strategy [29, 30] to raise an Ab response against the HIV-1 envelope glycoprotein gp120 in control and Bcl6FC mice. As shown in Fig. 4A, control and Bcl6FC

mice were injected i.m. with gp120-encoding DNA 3 times, 2 wk apart, for priming. 4 wk later, mice were received two gp120 protein booster immunizations via i.m. injection, 2 wk apart. 1 wk after the final injection, mice were sacrificed. In this repeated challenge model, we again did not see significant changes in the size of Tfh- or GCB-cell populations in Bcl6FC mice compared to control mice (data not shown). Unlike SRBC immunization, where Bcl6FC mice had a dramatic decrease of anti-SRBC IgG titers, anti-gp120 IgG titers were similar in the serum from control and Bcl6FC mice (Fig. 4B). As with other antigens, we saw enhanced levels of anti-gp120 IgA (Supp. Fig. 4). Because of the high titers of IgG produced in this model, we could assess the avidity of anti-gp120 IgG antibodies using a chaotropic reagent (NaSCN) displacement method (Fig. 4C). We observed a significant decrease in the avidity of anti-gp120 IgG antibodies in the Bcl6FC mice. Thus, Tfr cells have a positive role in maintaining anti-gp120 IgG Ab avidity in a multi-prime and multi-boost vaccine model.

Tfr cells regulate anti-dsDNA IgA Ab production in pristane induced lupus model

A major function of Tregs is the maintenance of self-tolerance, in order to prevent autoimmune diseases such as systemic lupus erythematosus (SLE). SLE patients exhibit high titers of pathogenic auto-antibodies and they usually have impaired Tregs, both in number and function [31, 32]. We hypothesized that loss of Tfr cells could make Bcl6FC mice more susceptible to SLE due to defective control of auto-Ab production. Control and Bcl6FC mice were injected i.p. with pristane to induce a lupus-like syndrome [33, 34]. 4 months after treatment, both control and Bcl6FC mice were healthy and without any sign of arthritis, a symptom sometimes seen in the pristane model. As with other immune challenge models, we did not find any changes in Tfh- or GCB-cell populations between control and Bcl6FC mice (data not shown). Control and Bcl6FC mice had similar titers of anti-dsDNA IgM and IgG antibodies in serum (Fig. 5A-B). However, we found significantly elevated anti-dsDNA IgA serum antibodies in Bcl6FC mice (Fig. 5C). Thus, in the pristane-induced lupus model, Tfr cells repress production of anti-dsDNA IgA, but not other isotypes of anti-dsDNA auto-Abs.

Tfr cells regulate cytokine production by Tfh cells

Cytokines produced by Tfh cells can promote GCB-cell proliferation and survival. Therefore we checked the production of cytokines (IFN- γ , IL-4, IL-10 and IL-21) known to be expressed by Tfh cells in splenic PD-1^{hi}, PD-1^{int} and PD-1^{neg} CD4⁺ T cells described previously [35]. Control and Bcl6FC mice were analyzed 10 days after SRBC immunization (Fig. 6A). As shown in Fig. 6B, PD-1^{hi} Tfh cells in Bcl6FC mice expressed significantly higher levels of several cytokines: IFN- γ , IL-10 and IL-21. There was no difference in IFN- γ and IL-21 expression by PD-1^{int} and PD-1^{neg} CD4⁺ T cells between control and Bcl6FC mice, however T cells generally produced more IL-10 in Bcl6FC mice (Fig. 6C-D). We observed a very similar cytokine phenotype in the Tfh cells in the gp120 DNA prime protein boost model (data not shown). Taken together, these data show Tfr cells specifically regulated IFN- γ and IL-21 production by Tfh cells, with little effect on cytokine production by PD-1^{int} or PD-1^{neg}CD4⁺ T cells.

Discussion

Here we have further characterized the phenotype and function of Tfr cells. We show that Tfr cells express much lower level of CD25 and higher level of inhibitory molecule TIGIT than non-Tfr Tregs. We have used the Bcl6^{fl/fl}Foxp3^{cre} (Bcl6FC) mouse strain to specifically delete Bcl6 in Tregs to analyze the regulation of Tfh cells and the GC reaction by Tfr cells. Unexpectedly, Tfr cells do not regulate the gross populations of Tfh or GCB cells, but regulate the normal Ab response following immune challenge, as shown with several models. Strikingly, Tfr cells can suppress the generation of IgA anti-dsDNA Abs in a pristane-induced autoimmune setting. Finally, Tfh cells consistently secrete more cytokines in the absence of Tfr cells, and we propose that this alters the cytokine milieu and affects the selection of GC B cells, leading to an abnormal Ab response.

The Bcl6FC mouse model is much more amenable to future in-depth experimentation than currently published approaches to specifically analyze Tfr-cell function. These mice can be bred in large numbers and will be useful to assess the role of Tfr cells in a wide variety of different immune conditions, from infection to autoimmunity. The Bcl6FC model stands in contrast to other approaches to analyze Tfr cells in which total Tregs were depleted after immunization [9, 10]. Total loss of Tregs can lead to wide-ranging immune activation effects that could impact the GC response by indirect means than by Tfr cells, such as by affecting early T cell proliferation before Tfh differentiation has occurred. Another approach used to study Tfr-cell function is to transfer T helper cells plus or minus Tfr cells into T cell-deficient animals, and analyze the Ab responses after immunization [9, 11, 13]. However, this approach has limitations in performing large numbers of such reconstitutions, and it is not clear whether such a reconstituted GC response is physiological. Another study deleted NFAT2 in Tregs or total CD4⁺ T cells to study the function of Tfr cells [14]. However the Tfr-cell deletion efficiency of this model was only about 50% and this study primarily focused on the up-regulation of CXCR5 of Tfr cells by NFAT2, without characterizing a more general Treg defect due to the deletion of NFAT2. Thus, the Bcl6FC mouse model shown here has much promise for further studies into Tfr cell function.

CD25 is one of the three subunits of IL-2 receptor whose signaling is critical for Treg differentiation, maintenance and survival [36, 37]. The down-regulated IL-2 signaling in Tfr cells due to their low expression of CD25 may explain the high rate of cell death of Tfr cells (Supp. Fig. 4). Of note, the remaining few Tfr cells in Bcl6FC mice have significantly higher levels of cell death than WT Tfr cells (Supp. Fig. 4). This indicates that Bcl6 may stabilize the survival of Tfr cells by counteracting the stress caused by a decreased level of IL-2 signaling. Importantly, unlike non-Tfr Tregs, CD25 is not a reliable surface marker for detecting Tfr cells.

As noted in the introduction, other studies observed expanded Tfh- and GCB-cell responses in the absence of Tfr cells. We propose that this is because in these earlier studies, total Tregs were depleted, which led to enhanced early Tfh priming or stronger responses during an ongoing GC response. One possibility with our Bcl6FC mouse model is that the Tregs cannot up-regulate Bcl6 and thus are stuck outside the B cell follicle. In theory, these “stuck” Tregs could more strongly suppress developing Tfh responses on the edge of the B

cell follicle, and this could then mask the effect of loss of Tfr cells on Tfh cells within the GC, and balance out the stronger Tfh/GC responses that would be otherwise seen without Tfr cells. While this is a possibility, we show in Figure 6C that PD1- and CXCR5-intermediate Foxp3- T cells, which can be considered as early- or pre-Tfh cells, do not have a decreased expression of cytokines consistent that would be consistent with increased suppression by Tregs. Also, in the Bcl6Fc mice, there is not a decreased percentage of PD1- and CXCR5-intermediate Foxp3- T cells (early/pre-Tfh cells) that would be consistent with increased suppression by Tregs at this stage (Supp. Fig. 5). Another possibility is that the few Tfr cells left in the Bcl6FC mice are enough to prevent abnormal outgrowth of Tfh and GCB cells. While this is hard to rule out completely, we doubt that any other system used previously was able to delete Tfr cells more completely than we have here, and further, the few remaining Tfr cells in Bcl6FC mice are undergoing increased apoptosis (Supp. Fig. 5).

Previous studies on Tfr cells led to conflicting results on the precise role of Tfr cells in controlling Ab responses [9-11, 15]. In general, previous work was in agreement with the idea that Tfr cells suppress the size of the GC response. However, the previous studies came to different conclusions on the effect of Tfr cells on the Ab response. These different results reflected the different systems used to analyze Tfr function, which had limitations, as discussed above. In our study, we used multiple immunization models, immunization with SRBC, NP-KLH/Alum, HIV gp120 DNA prime protein boost vaccination and pristane treatment. In all of these models, loss of Tfr cells did not lead to an increase of GCB-cell populations at any of the time-points we examined. Unexpectedly, we observed a decrease in Ag-specific IgG production and an increase in Ag-specific IgA production in the Bcl6FC mice, showing a novel role for Tfr cells in controlling isotype switch of Abs. We currently do not know whether in the absence of Tfr cells, there is a deviation of the normal IgG response to an IgA response, or whether there are separate defects causing these opposite effects on Ig isotype. In general, the decrease in IgG titer is stronger than the increase in IgA titer, indicating that there is not a simple compensating switch from IgG switching to IgA. Likely, the altered cytokine expression we observe from Tfh cells from Bcl6FC mice contributes to the decreased IgG and increased IgA. Answering this question will require a great deal of additional research.

In the HIV-1 gp120 model [29, 30], we observed a decrease in the affinity of anti-SRBC Abs. One possible explanation for this result, as pointed out in Linterman *et al* [10], is the expansion of non-SRBC-specific or low affinity Ag-specific GCB cells in the absence of Tfr cells. In this model, Bcl6FC mice develop normal titers of anti-gp120 IgG Abs but with significantly decreased avidity. Probably due to the long term repeated challenge nature of this vaccination strategy, the Bcl6FC mice were able to produce normal titers of Ab but still could not produce highest affinity Abs due to abnormal GC B cell selection in the absence of Tfr cells. These findings suggest that monitoring Tfr cells during the course of vaccination may help predict vaccine efficacy in producing high affinity Abs.

TIGIT is an important co-inhibitory molecule on Tregs for controlling pro-inflammatory Th1 and Th17 cells [24]. We demonstrate here that Tfr cells express high levels of TIGIT. In the absence of Tfr cells, there is therefore a lack of TIGIT-mediated immune suppression

from Tfr cells in GCs in Bcl6^{FC} mice. Accordingly, we observed increases in production of IFN- γ , a Th1 cytokine, and IL-21, a Th17 or Tfh cytokine, by Tfh cells in these mice after SRBC immunization or gp120 vaccination. Tfh cells themselves express multiple negative immune checkpoint regulators such as, PD-1, BTLA and TIGIT [18, 22, 23], which may relate to why we did not find any abnormality in the size of Tfh cell population. Competition for IL-21, a known B cell growth factor, could enhance survival of high affinity clones throughout the GC response [38, 39]. The increase of production of IL-21 by Tfh cells in GCs in Bcl6^{FC} mice could provide enhanced survival signal to non-antigen specific or low affinity B cell clones, leading to the decrease in titers of anti-SRBC IgG Abs and in the avidity of anti-gp120 IgG Abs.

Tregs are important for the maintenance of the immunological tolerance and self-/non-self-discrimination [37]. One of the hallmarks of SLE is the generation of anti-dsDNA auto-Abs in which the GC response plays important role [40]. The discovery of Tfr cells gives us a great opportunity to better understand the regulation of GC response in SLE. We used a pristane-induced lupus model with our Bcl6^{FC} mice. Either the genetic background of our mice (C57Bl/6) is not ideal for pristane treatment [41] or the 4 month incubation period after pristane treatment was not long enough, since neither control mice nor Bcl6^{FC} mice developed severe disease symptoms. However a GC B cell response and antibodies against dsDNA did arise from pristane treatment. We observed the significant increase of anti-dsDNA IgA titer in the absence of Tfr cells indicating a critical role of Tfr cells in controlling autoimmune GC response in SLE. The increase in production of IFN- γ and IL-21 by Tfh cells in Bcl6^{FC} mice could be an additional reason for elevated IgA anti-dsDNA Ab titer, since both IFN- γ and IL-21 are involved the generation of auto-antibodies [42, 43]. In the clinic, the elevated level of IgA anti-dsDNA Abs is associated with kidney and joint abnormalities in SLE patients [44, 45]. Regulation of the GC response by the Tfr cells in SLE patients or other autoimmune diseases is an important area for future investigation.

Overall, our study helps to expand on, clarify and reconcile some of the previous findings on Tfr cells. We show Tfr cells can promote normal levels and types of Ab responses against foreign antigens, help maintain Ab affinity and regulate generation of anti-self antigen Abs in autoimmune responses. Our Tfr cell-specific deletion mouse model should be further used for understanding the role of Tfr cells in vaccination and in controlling pathogenic Ab responses.

Materials and Methods

Mice

Bcl6^{fl/fl} mice were previously described [25]. Foxp3-YFP-cre mice [46] were obtained from Jackson labs. Genotyping for the floxed Bcl6 allele was performed as described [25]. Genotyping for Foxp3-YFP-cre allele was according to PCR protocols from Jackson labs. Bcl6^{fl/fl} mice were crossed to Foxp3-YFP-cre mice. Control mice for Foxp3-cre Bcl6^{fl/fl} (Bcl6^{FC}) conditional KO mice were Foxp3-cre Bcl6^{+/+} (wild-type [WT]) mice. Mice were bred under specific pathogen-free conditions at the laboratory animal facility at IUSM and

were handled according to protocols approved by the IUSM Animal Use and Care Committee.

Immunization

For sheep red blood cells (SRBC) immunization, mice were i.p. injected with 1×10^9 SRBC (Rockland Immunochemicals) and were sacrificed at the indicated day. For KLH (Sigma-Aldrich) immunization, 100 ug KLH was mixed with Imject Alum (Pierce) and the mixture was injected i.p. For gp120 DNA prime-protein boost vaccine immunization as previous reported [29, 30], gp120 DNA vaccine construct in pJW4303 vector and gp120 protein produced from Chinese Hamster Ovary (CHO) cells were used. Mice were primed by i.m. injections of 100 ug of gp120 construct every two weeks for three times. Four weeks after the third DNA immunization, mice were boosted with 10 ug gp120 protein plus Alum mixture every two weeks for twice. Mice were sacrificed one week after the second gp120 protein boost. For both DNA and protein immunizations, a total of 100 ul was injected, 50 ul per hind leg.

Pristane administration

WT and Bcl6^{FC} mice were given a single i.p. injection of 0.5 ml Pristane (Sigma-Aldrich) at 12 weeks of age. After 4 months, mice were sacrificed. The presence of anti-dsDNA antibodies in the serum was tested by ELISA, and spleens were taken out for further flow cytometry analysis.

Flow cytometry reagents

Anti-CXCR5 (2G8), GL7 (GL7), and anti-IL-4 (11B11) Abs were from BD Biosciences. Fixable viability dye, anti-CD38 (90), anti-TIGIT (GIGD7), anti-IL-21 (mhalx21), and anti-Foxp3 (FJK-16s) Abs were from eBioscience. Anti-CD4 (GK1.5), anti-B220 (RA3-6B2), anti-PD1 (29F.1A12), anti-CD25 (PC61), anti-IL-10 (JES5-16E3), Annexin V and anti-IFN- γ (XMG1.2) were from Biolegend.

Cell staining for flow cytometry

Cell suspension from spleen cells were firstly incubated with anti-mouse CD16/CD32 (Bio X Cell) for 5 min at room temperature, followed with surface staining of indicated markers and viability staining. For intracellular staining, after surface staining, cell were fixed and stained with Abs against intracellular proteins by following Foxp3 fixation kit (eBioscience) protocol. Cell events were collected on LSRII flow cytometer (BD Biosciences) and analyzed by Flowjo® software.

Intracellular cytokine staining

2×10^6 cells were stimulated with PMA (75 ng/ml) plus ionomycin (1 ug/ml) for 5 hours in DMEM with 10% FBS, then fixed and stained for indicated cytokines as described [35]. GolgiStop and GolgiPlug (BD Biosciences) were added with the stimulation to inhibit cytokine release.

Preparation of SRBC membrane antigens for ELISA

All procedures were carried out at 4°C. After each centrifugation, the supernatant were removed by vacuum. SRBC (Rockland Immunochemicals) were centrifuged at 13,000g for 10 min. The pellet was re-suspended in lysis buffer (0.05 M Tris-HCL with 0.1 mM EDTA, pH 7.6), vortexed thoroughly, and then centrifuged again. This process was repeated 7 to 10 times to remove all visible traces of hemoglobin. Before last centrifugation, the pellet was re-suspended and passed through a 40 µm cell strainer. After the final wash, the pellet from 5 ml SRBC was re-suspended in 500 µl 0.1% SDS and stored at -20 °C.

Anti-SRBC, anti-dsDNA and anti-gp120 Ab titers analysis

Anti-SRBC IgG/IgM, and anti-dsDNA IgA/IgG/IgM titers were determined by ELISA, as previously reported [25]. Briefly, 96-well Nunc-Immuno plates (Sigma-Aldrich) were coated with SRBC membrane antigens or salmon sperm DNA passed through 45 µm filter (Invitrogen) overnight at 4°C. Wells were blocked with 1% BSA for 1.5 hours. Diluted serum samples were applied. ELISA plates were incubated for 2 hours at room temperature for anti-SRBC Ab detection, or incubated for 1 hour at 37°C for anti-dsDNA Ab detection. Peroxidase-conjugated anti-mouse IgA, anti-mouse IgG or anti-mouse IgM Abs (Sigma-Aldrich) was used as secondary Ab. Anti-dsDNA ELISA plates were washed extensively after each incubation to minimize high background signals. Anti-gp120 IgG titers were measured by ELISA as previously described [29, 30]. Anti-gp120 Ab avidity was measured via the NaSCN displacement method, as previously reported [29, 30].

Statistical analysis

All data analysis was done in GraphPad Prism software. Unless otherwise stated, a Student *t* test or ANOVA with a Tukey post hoc analysis were used. Only significant differences ($p < 0.05$) are indicated in figures.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

This work was supported by NIAID grants R03 AI110987, R21 AI099825, R21 AI113523 and R56 AI112398 to A.L.D.

List of abbreviations

Tfh	T follicular helper cells
Tfr	T follicular regulatory cells
Treg	regulatory T cells
Tconv	T conventional
GC	germinal center

SRBC	sheep red blood cell
Bcl6FC	Bcl6 ^{fl/fl} Foxp3 ^{cre}

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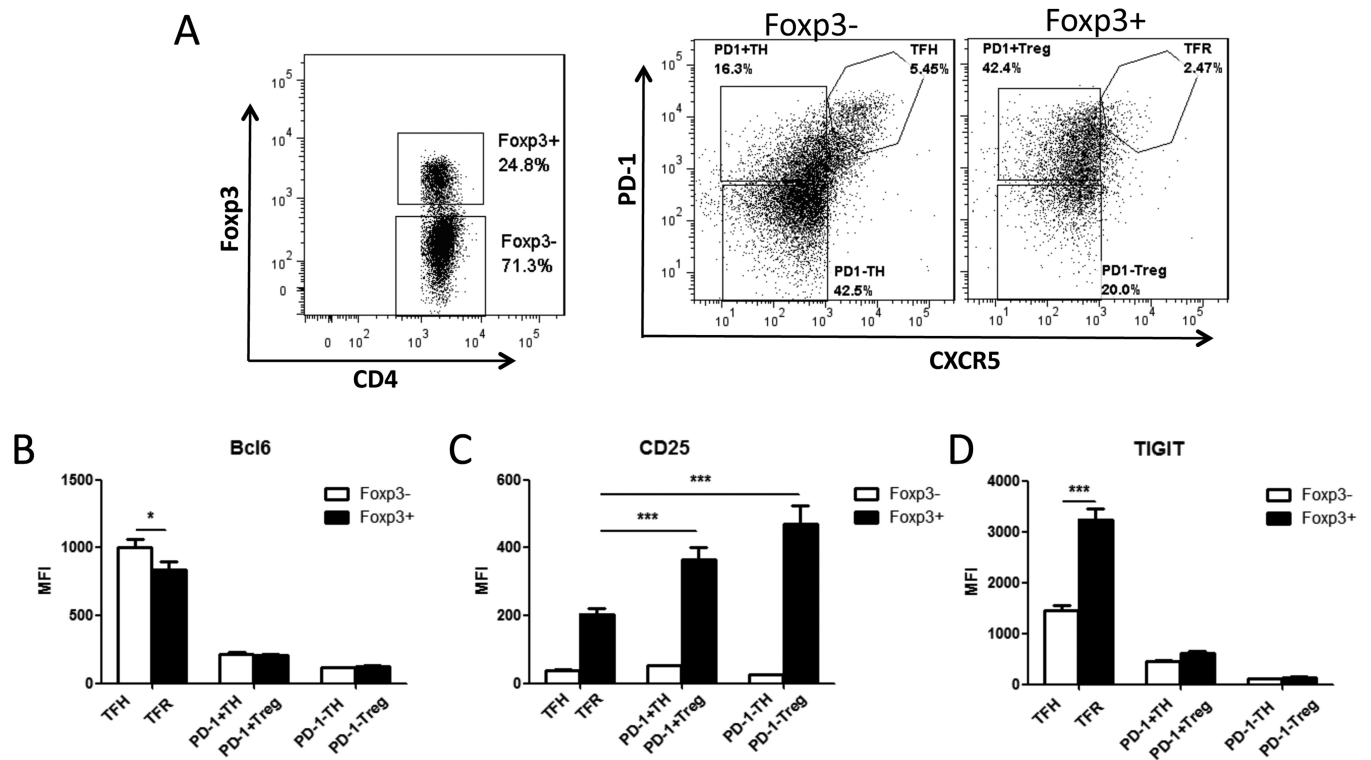


Figure 1. Bcl6, CD25 and TIGIT levels in various conventional and regulatory T cell subsets
 Splenic CD4⁺ T cells from WT mice immunized with Sheep Red Blood Cells (SRBC) 7 d before were studied by flow cytometry. (A) Foxp3⁺ and Foxp3⁻ CD4⁺ T cells are gated as in the flow plot (left). In Foxp3⁻ conventional CD4⁺ T (Tconv) cells, Tfh cells, PD-1+TH cells and PD-1- TH cells are defined as CXCR5^{hi}PD-1^{hi}, CXCR5^{neg}PD-1⁺ and CXCR5^{neg}PD-1^{neg}, respectively. In Foxp3⁺ regulatory CD4⁺ (Treg) cells, Tfr, PD-1+Treg and PD-1- Treg cells are gated on CXCR5^{hi}PD-1^{hi}, CXCR5^{neg}PD-1⁺ and CXCR5^{neg}PD-1^{neg}, respectively (right). Bcl6 (B), CD25 (C) and TIGIT (D) mean fluorescent intensity (MFI) of Tfh, PD-1+TH, PD-1- TH, Tfr, PD-1+Treg and PD-1- Treg cells. Data shown as mean \pm SEM, n = 4-5. * p < 0.05, ** p < 0.01, *** p < 0.001 (ANOVA). Data are representative of two independent experiments with similar results.

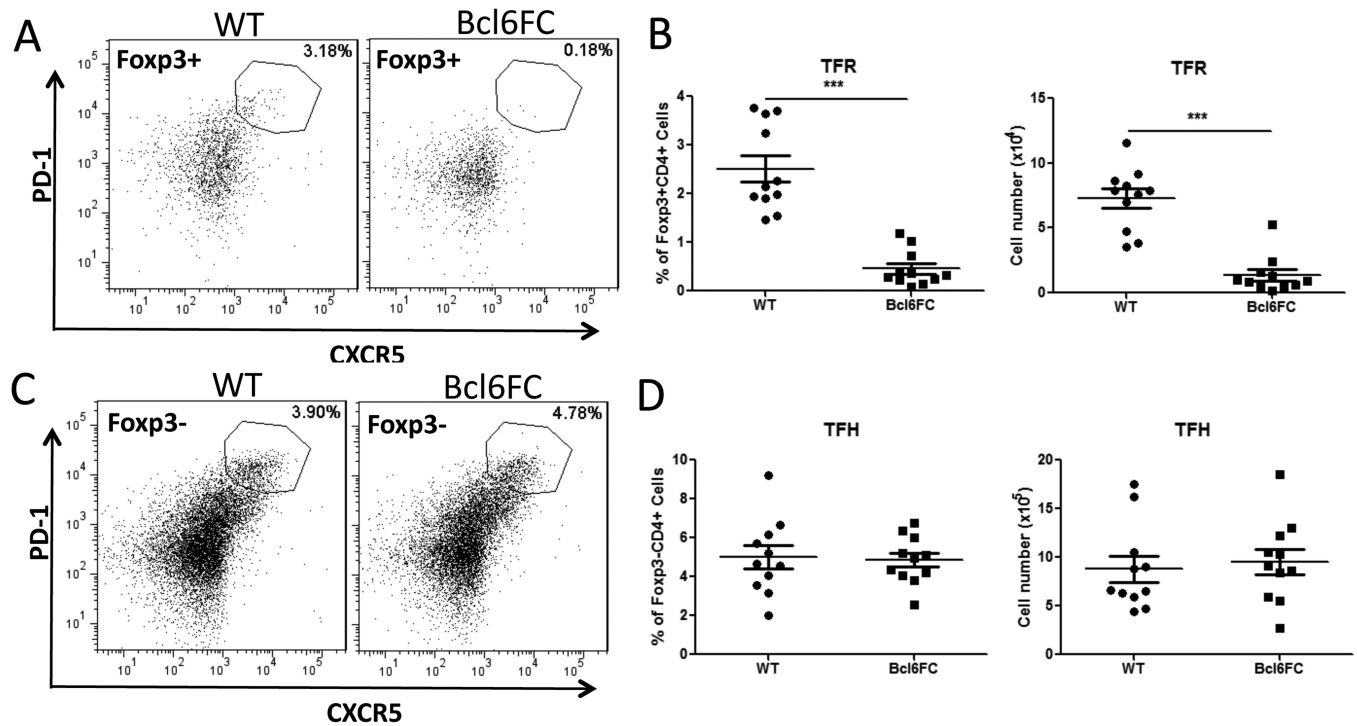


Figure 2. Loss of Tfr cells in Bcl6FC mice does not affect the size of Tfh cell population following SRBC immunization

Control and Bcl6FC KO mice were immunized with SRBC by i.p. injection. 10 day post immunization (dpi), spleens were isolated for flow cytometric analysis. (A) Tfr cells are defined as Foxp3⁺CXCR5^{hi}PD-1^{hi}. (B) Tfr cell percentage in CD4⁺Foxp3⁺ T cells and Tfr cell number. (C) Tfh cells are defined as Foxp3⁻CXCR5^{hi}PD-1^{hi}. (D) Tfh cell percentage in CD4⁺Foxp3⁻ T cells and Tfh cell number. Data shown as mean \pm SEM, n = 11. ** $p < 0.01$ (student t test). Data are pooled from three independent experiments.

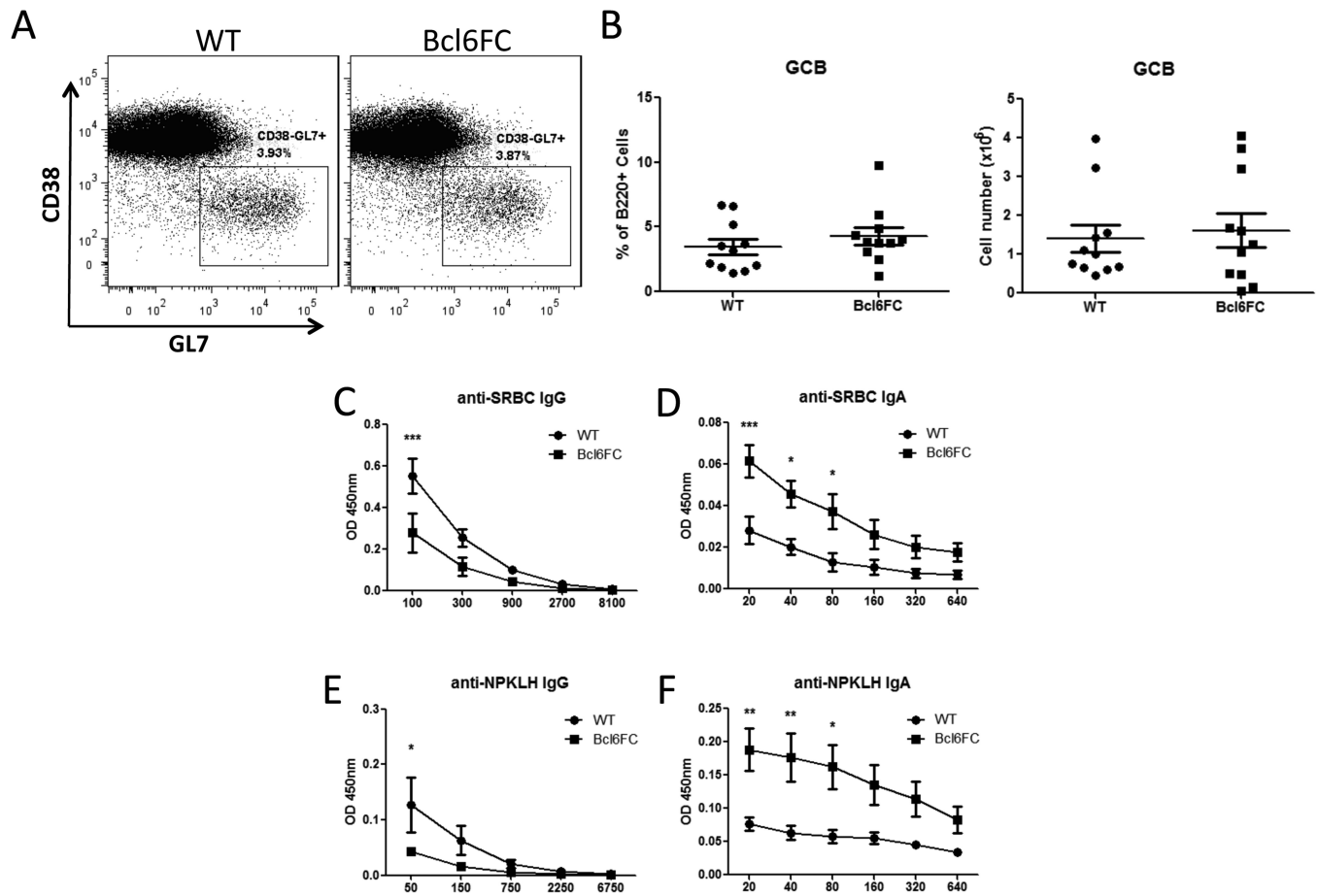


Figure 3. Tfr cells do not regulate the size of GCB cell population, but contribute to the generation of antigen specific IgG antibodies by restraining antigen specific IgA antibodies
Control and Bcl6FC KO mice were immunized with SRBC by i.p. injection. Spleens were isolated for flow cytometric analysis at 10 dpi. (A) GCB cells are defined as B220⁺CD38⁻GL7⁺ in flow plot. (B) GCB cell percentage in B220⁺ cells and cell number. Serum samples from control and Bcl6FC mice were also collected. (C) Anti-SRBC IgG, (D) anti-SRBC IgA titers. The X-axis shows the dilution factors. (E, F) Control and Bcl6FC mice were immunized with NP-KLH in Alum. Serum samples were collected at 25 dpi. (E) Anti-NPKLH IgG, (F) anti-NPKLH IgA titers. The X-axis shows the dilution factors. Graphs show mean \pm SEM, (A-B) $n = 11$, (C-D) $n = 4$, (E-F) $n = 4-6$. ** $p < 0.01$, *** $p < 0.001$ (student t test for B, two-way ANOVA for C-F). For A-B, data are pooled from three independent experiments. For C-F, data are representative of three independent experiments with similar results.

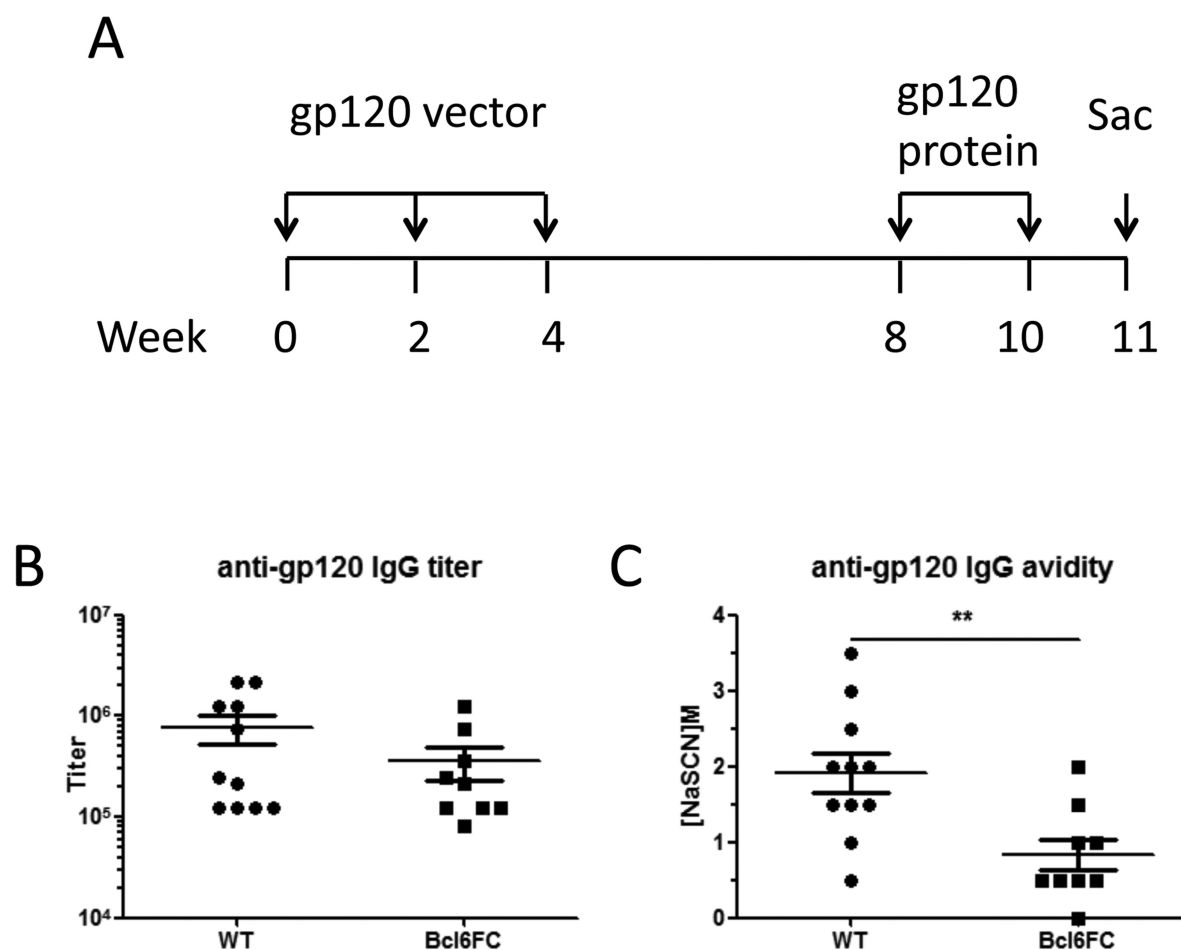


Figure 4. Decreased antibody avidity in the absence of Tfr cells in the DNA prime-protein boost gp120 vaccine model

(A) Experimental setup of gp120 DNA prime and protein boost immunization. Control and Bcl6FC mice were primed i.m. with gp120-encoding DNA 3 times, 2 wk apart. 4 wk later they were given 2 booster injections of gp120 protein, 2 wk apart. Spleen and serum samples from control and Bcl6FC mice were collected one week after the final booster. (B) Serum anti-gp120 specific IgG titers measured by ELISA and (C) avidity of gp120- specific IgG antibodies measured by NaSCN displacement method are shown. Data shown as mean \pm SEM, $n = 9-11$. ** $p < 0.01$ (student t test). Data are pooled from two independent experiments.

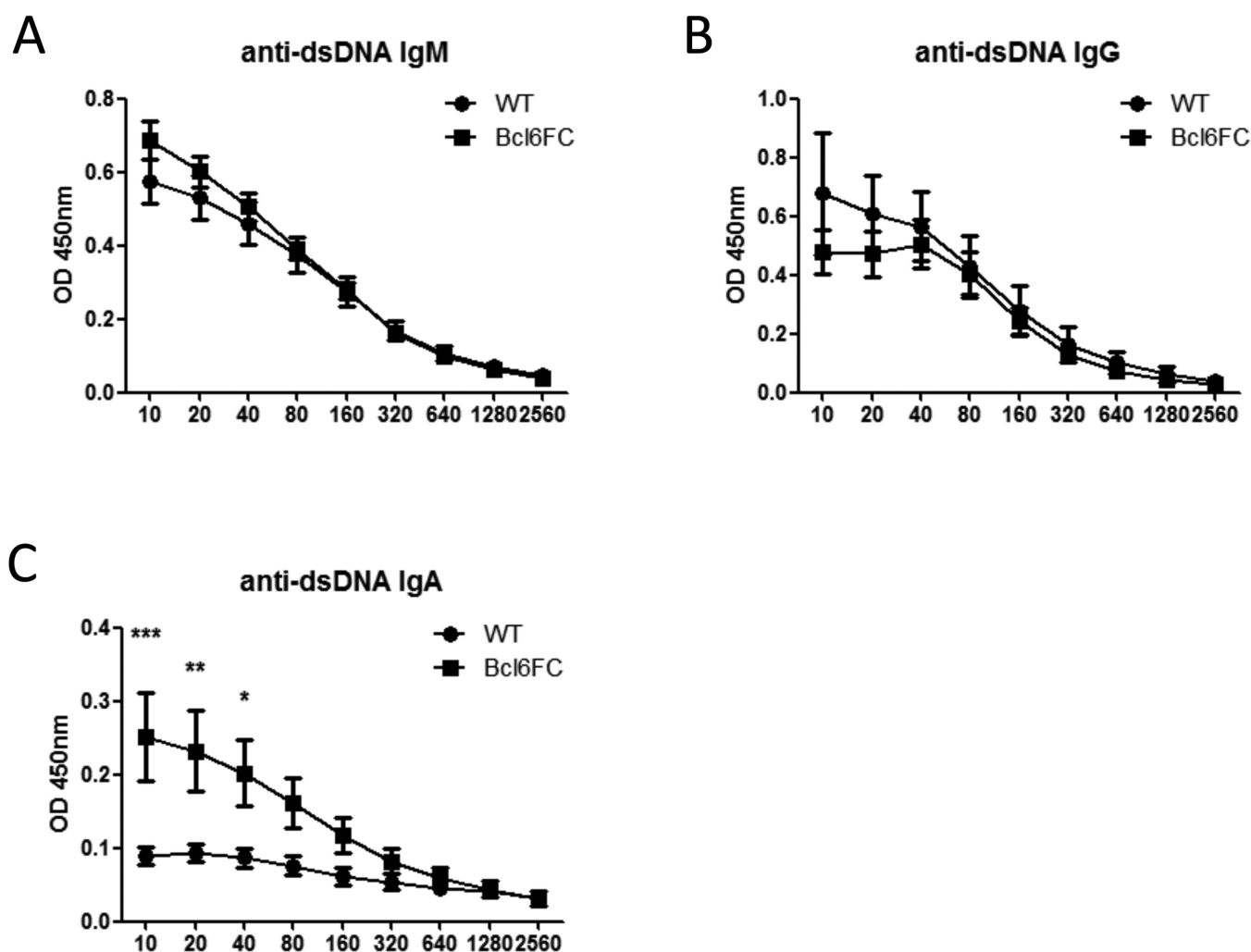


Figure 5. Tfr cells regulate the induction of anti-dsDNA IgA antibodies in Pristane-induced lupus model

Four months after single 0.5 ml pristane i.p. injection, serum samples were collected from control and Bcl6FC mice for anti-dsDNA antibody detection by ELISA. (A) Anti-dsDNA IgM, (B) anti-dsDNA IgG and (C) anti-dsDNA IgA titers are shown. The X-axis labels are dilution factors. Data shown as mean \pm SEM, $n = 6-7$. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ (two-way ANOVA). Data are pooled from two independent experiments.

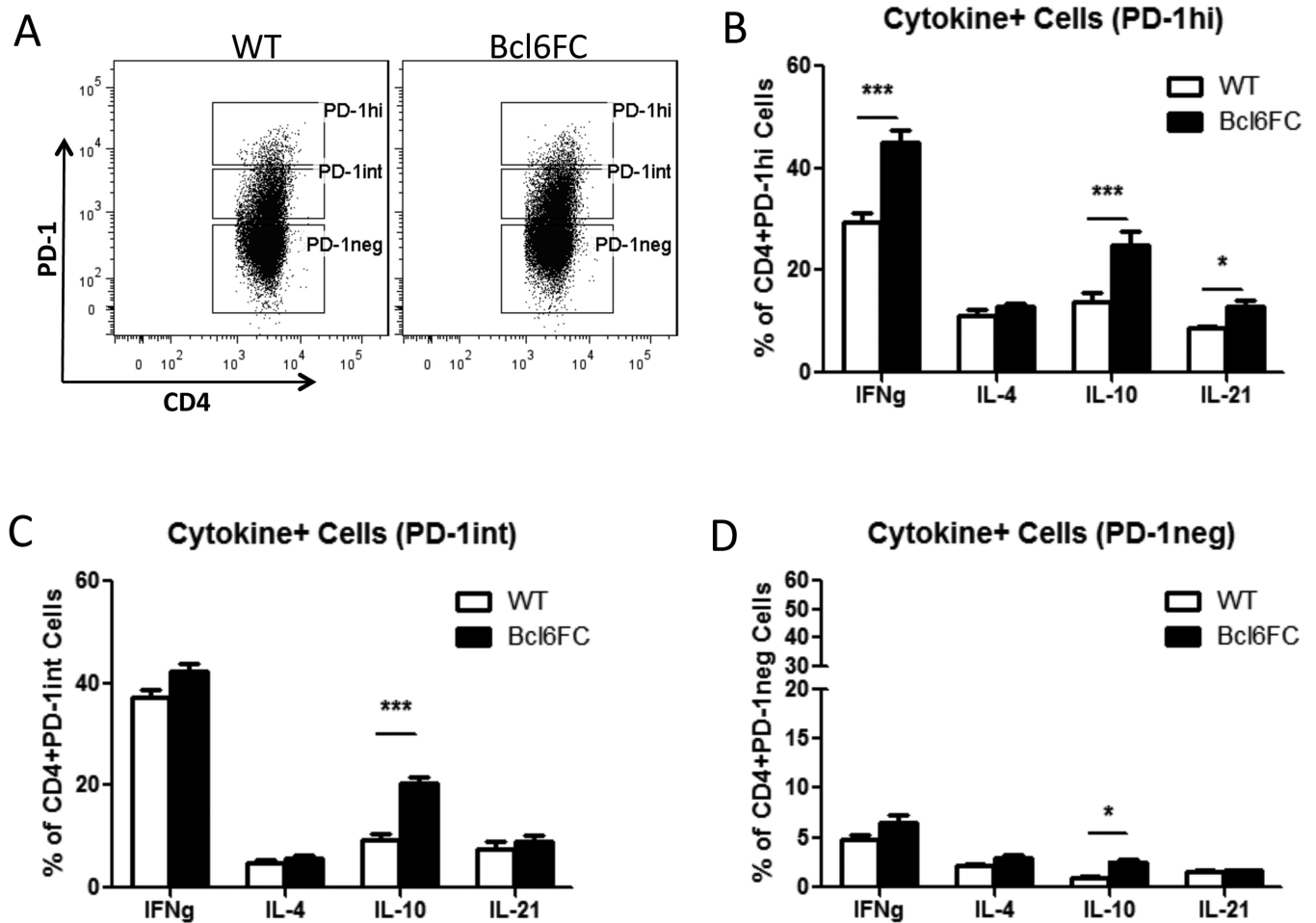


Figure 6. Tfr cells regulate IFN- γ , IL-10 and IL-21 production in Tfh cells
 (A) PD-1^{hi}, PD-1^{int} and PD-1^{neg} CD4⁺ T cells gates. Tfh cells are defined as CD4+PD-1^{hi} cells. Percentages of cytokine-producing (IFN- γ , IL-4, IL-10 and IL-21) in CD4+PD-1^{hi}Tfh (B), CD4+PD-1^{int}T (C) and CD4+PD-1^{neg}T cells (D) from control and Bcl6FC mice 10 days after SRBC immunization measured by ICS are shown. Data shown as mean \pm SEM, $n = 11$. * $p < 0.05$, *** $p < 0.001$ (student t test). Data are pooled from three independent experiments.